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Glycosphingolipids are required for sorting melanosomal proteins in the Golgi complex

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Although glycosphingolipids are ubiquitously expressed and essential for multicellular organisms, surprisingly little is known about their intracellular functions. To explore the role of glycosphingolipids in membrane transport, we used the glycosphingolipid-deficient GM95 mouse melanoma cell line. We found that GM95 cells do not make melanin pigment because tyrosinase, the first and rate-limiting enzyme in melanin synthesis, was not targeted to melanosomes but accumulated in the Golgi complex. However, tyrosinase-related protein 1 still reached melanosomal structures via the plasma membrane instead of

the direct pathway from the Golgi. Delivery of lysosomal enzymes from the Golgi complex to endosomes was normal, suggesting that this pathway is not affected by the absence of glycosphingolipids. Loss of pigmentation was due to tyrosinase mislocalization, since transfection of tyrosinase with an extended transmembrane domain, which bypassed the transport block, restored pigmentation. Transfection of ceramide glucosyltransferase or addition of glucosylsphingosine restored tyrosinase transport and pigmentation. We conclude that protein transport from Golgi to melanosomes via the direct pathway requires glycosphingolipids.

Introduction

Glycosphingolipids consist of a carbohydrate moiety that is attached to ceramide, a lipid anchor with two hydrophobic tails. Although space-filling models suggest that glycosphingolipids and the more abundant glycerophospholipids may have similar properties, this clearly is an oversimplification. Whereas glycerophospholipids consist of two fatty acyl chains esterified to glycerol, glycosphingolipids have a single fatty acid conjugated via an amide bond to the C2 position of the sphingoid base. In addition, ceramides contain free hydroxyl groups close to the carbohydrate moiety, and their fatty acid tends to be longer and more saturated than those at the glycerol C2 position of the glycerophospholipids. Due to these compositional differences, the affinity between glycosphingolipids is usually higher than between glycerophospholipids.

The most simple glycosphingolipids are glucosylceramide (GlcCer)* and galactosylceramide (GalCer). GlcCer occurs in all mammalian cells and serves as the precursor for a large number of complex glycosphingolipids. In contrast, GalCer is expressed in specialized cells and can be sulfated or galactosylated but is generally not further modified. Several cellular membranes contain high glycosphingolipid levels. In myelin and the apical membrane of some epithelial cells, glycosphingolipid may constitute up to 20–35 mol% of total lipid. The high glycosphingolipid content of these plasma membranes is thought to mediate their insulating and protective functions.

The diverse chemical structure of complex glycosphingolipids suggests that they are involved in cell–cell and cell–substratum interactions (Hakomori et al., 1998). Although such interactions have been the subject of many studies, surprisingly little is known about their intracellular functions. Importantly, sphin-

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P. van der Sluijs and G. van Meer contributed equally to this work.

Key words: glycosphingolipid; protein sorting; melanosome; tyrosinase; TRP-1

*Abbreviations used in this paper: CGlCT, ceramide glucosyltransferase; CGalT, ceramide galactosyltransferase; L-DOPA, L-3,4-dihydroxyphenylalanine; GalCer, galactosylceramide; GlcCer, glucosylceramide; GlcSph, glucosylsphingosine; Man6P, mannose 6-phosphate; MPR, mannose 6-phosphate receptor; NB-DNJ, *N*-butyldeoxynojirimycin; TRP, tyrosinase-related protein.

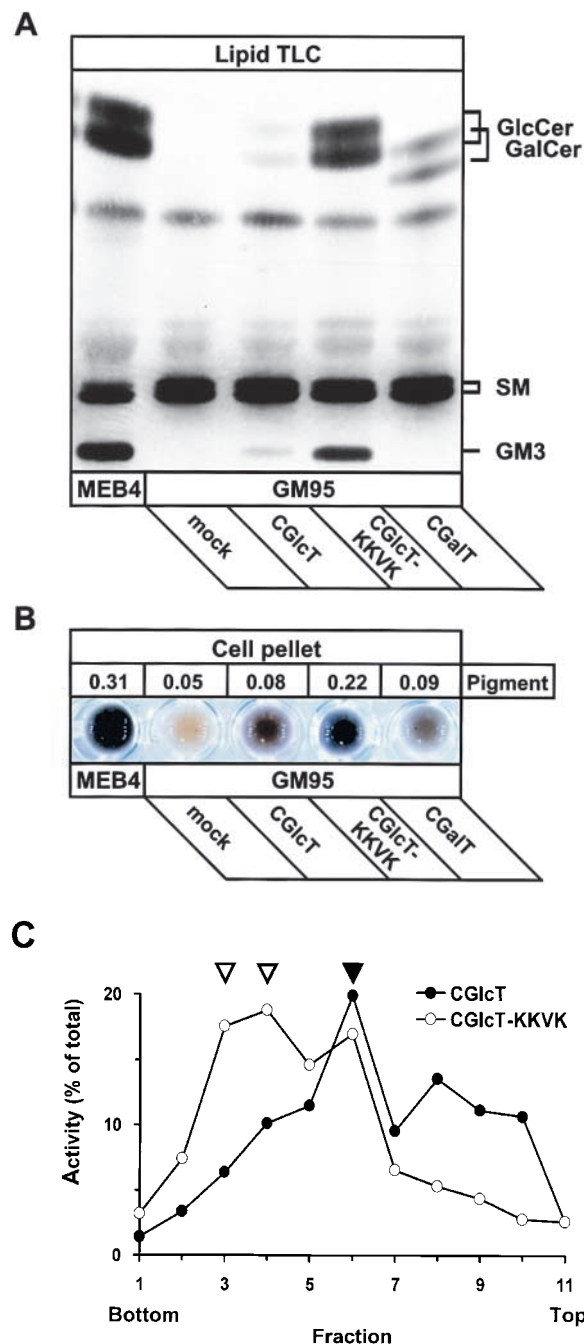


Figure 1. Sphingolipid composition and pigmentation of melanoma cells. (A) Cells were labeled with [^3H]sphingosine for 2 d. Lipids were separated by TLC and visualized by fluorography. Spots were scraped and quantified by liquid scintillation counting. Incorporation of [^3H] (10^3 dpm) in MEB4 cells, GM95 cells transfected with empty vector (mock), CGlcT, and CGlcT-KKVK, respectively. GlcCer, 51, not detectable (ND), 0.7, and 25; GM3, 22, ND, 1.2, and 6; sphingomyelin (SM), 47, 84, 96, and 54. GM95 cells transfected with CGaIT: GalCer, 6.5; sphingomyelin, 97, $n = 4$; background: 0.2; ND, < 0.4 . (B) Cells were scraped, pelleted in a microtiter plate, and photographed. Next, cell pellets were solubilized, and pigment was measured colorimetrically (A_{475} /mg protein; representative experiment in quadruplicate, SD = 0.01; unpigmented CHO cells as a measure of light scattering, 0.03). (C) Distribution of CGlcT activity after fractionating a postnuclear supernatant of GM95-CGlcT (●) or GM95-CGlcT-KKVK (○) on 0.7–1.5 M linear sucrose gradients. Each profile is the mean of two gradients. Total CGlcT activity was sevenfold lower in GM95-CGlcT. The peak fractions of calreticulin and sphingomyelin synthase are indicated by ∇ and \blacktriangledown , respectively.

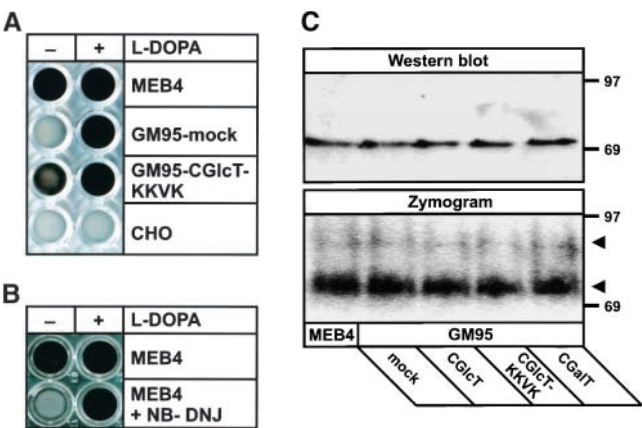


Figure 2. Pigmentation machinery of MEB4 and GM95 cells. (A) Cells were incubated with or without 1 mM L-DOPA for 3 h at 37°C, scraped, and pelleted into a microtiter plate. (B) Cells were treated as under A, but MEB4 cells were pretreated for 3 d with 0.5 mM NB-DNJ to inactivate tyrosinase. NB-DNJ prevents successful glycosylation of tyrosinase and its folding to the active form (Petrescu et al., 1997). (C) Equal amounts of protein were resolved by SDS-PAGE and detected by Western blotting using anti-pep7. In gel DOPA oxidase, activity was measured as described in Materials and methods. DOPA oxidase activity in the higher molecular weight band corresponds to TRP-1.

golipids and in particular glycosphingolipids have the propensity to cluster in an environment of other lipids (Brown and London, 2000). Therefore, some of their functions may be explained by the ability to form lateral microdomains with physicochemical properties that are distinct from those of the bulk membrane (Hakomori et al., 1998; Brown and London, 2000). In 1988, we proposed that lateral domains of glycosphingolipids in the TGN are involved in the sorting of membrane proteins (Simons and van Meer, 1988). The ubiquitous expression of glycosphingolipids suggests that they exert organizing functions in all eukaryotic cells (Holthuis et al., 2001).

Knock-out mice with null alleles for ceramide glucosyltransferase (CGlcT) lack GlcCer-derived glycolipids and die at embryonic day 7.5 (Yamashita et al., 1999). However, individual embryonic cells derived from these mice are viable, which shows that glycosphingolipids are essential for multicellular organisms. Nevertheless, the intracellular functions of glycosphingolipids remain to be defined, since glycosphingolipid-deficient GM95 mouse melanoma cells appear to be perfectly viable (Ichikawa et al., 1994). Thus, neither in vivo nor in vitro models for glycolipid deficiency, so far, suggested a function for these lipids in the individual cell. We now report that GM95 cells do not synthesize melanin pigment as a result of which these cells are white, whereas the parental MEB4 B16 melanoma cells are black. This dramatic phenotype is caused by defective intracellular transport of melanosomal proteins from the Golgi complex to melanosomes in the absence of glycosphingolipids.

Results

Sphingolipid composition and pigmentation of the cell lines

When passaging the glycolipid-negative GM95 cells and the parental MEB4 cells, we noticed a striking difference between

Table I. Oxidation of tyrosine to L-DOPA by cultured cells

Cells	L-DOPA pmol/10 ⁶ cells
CHO	ND
MEB4	76 ± 32
GM95-mock	11 ± 2
GM95-CGLcT-KKVK	70 ± 33
GM95-tyrosinase-TM6	79 ± 38

L-DOPA was measured in cells (3 d) and medium as described in Materials and methods. Since L-DOPA is unstable at pH 7.4, the assay likely yields underestimates of cellular L-DOPA concentrations. GM95 cells were transfected with empty vector (mock), CGLcT with ER retrieval signal (CGLcT-KKVK), and tyrosinase with extended transmembrane domain (tyrosinase-TM6; Fig. 5). Considerable amounts of L-DOPA were found in medium of MEB4, GM95-CGLcT-KKVK, and GM95-tyrosinase-TM6 cells (1.2 ± 0.4 nmol/10⁶ cells), whereas L-DOPA was not detectable in medium of CHO or GM95 cells (<0.01 nmol/10⁶ cells). ND, not detectable: <0.5 pmol/10⁶ cells. Values are from two independent experiments.

the cell lines. Whereas the MEB4 cell pellet was black, the pellet of GM95 cells was white. The degree of pigmentation of the MEB4 cells increased fourfold when 1 mM L-tyrosine was added to the growth medium (Slominski et al., 1988; Smit et al., 1997), confirming that the black color of MEB4 melanoma cells is due to the pigment melanin of which L-tyrosine is the precursor. The GM95 cells remained white in the presence of L-tyrosine and thus did not synthesize melanin (Fig. 1).

The GM95 cell line has been selected as a mutant of MEB4 that shows no surface expression of the glycolipid GM3 (sialyl-galactosyl-GlcCer) due to a lack of CGLcT activity (Ichikawa et al., 1994). Lipid analysis confirmed (Fig. 1 A) that MEB4 cells produced GlcCer, LacCer (galactosyl-GlcCer), and GM3, whereas GM95 cells synthesized no glycosphingolipids. Partial restoration of glycolipid synthesis was achieved by stable transfection of the GM95 cells with human CGLcT cDNA, which also returned pigmentation as shown in Fig. 1 B. It is unclear why the human enzyme only minimally restores GlcCer synthesis in these mouse cells, but both glycolipid synthesis and pigmentation increased dramatically when GM95 cells were transfected with a cDNA encoding human CGLcT with the ER retrieval signal KKVK, where CGLcT localizes to the site where its substrate ceramide is synthesized (Fig. 1, A and B). In this cell line, CGLcT was to a large extent relocated from the Golgi to the ER as shown by subcellular fractionation (Fig. 1 C; Burger et al., 1996). Also, transfection with ceramide galactosyltransferase (CGalT) cDNA restored pigmentation (Fig. 1 B). CGalT, which synthesizes GalCer and galactosyldiglycerides in the ER of specialized cells (Burger et al., 1996; Sprong et al., 1998) is not related to CGLcT. Thus, pigmentation correlated directly with the synthesis of glycolipids.

GM95 cells contain active tyrosinase but do not make L-3,4-dihydroxyphenylalanine

To define the molecular basis of the pigmentation defect in GM95 cells, we next measured the oxidation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosinase *in vivo*, the first and rate-limiting step in melanin synthesis in the melanosome. In contrast to MEB4 cells, GM95 cells produced very little L-DOPA (Table I). Subsequent oxidation of L-DOPA and polymerization into melanin were re-

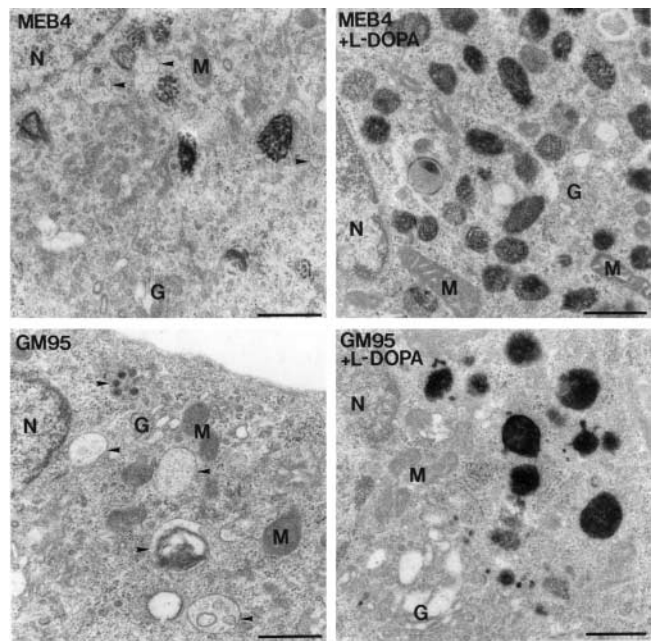


Figure 3. **EM of epon sections.** MEB4 and GM95 cells were incubated for 3 h at 37°C in the absence or presence of 1 mM L-DOPA before fixation. Comparable regions of the cell were selected for illustration. In MEB4 cells, melanosomes are readily recognizable by their dark melanin content. The arrowheads point to endosome-like compartments with the same size and shape as melanosomes but which lack melanin pigment. Incubation of MEB4 cells with L-DOPA increased the number of melanin-containing compartments. GM95 cells contain many endosome-like compartments but lack melanin. Incubation of GM95 cells with L-DOPA induces the appearance of pigmented organelles, suggesting that the compartments involved in melanin formation are present in these cells but that the production of melanin is impaired. G, Golgi; N, nucleus; M, mitochondrion. Bars, 500 nm.

tained in GM95 cells, since the cells turned black after incubation with exogenous L-DOPA (Fig. 2 A). Therefore, the pigmentation defect in GM95 cells appeared to be at the level of tyrosinase. Indeed, treatment of MEB4 cells with *N*-butyldeoxynojirimycin (NB-DNJ), a potent inhibitor of the ER α -glucosidases and of maturation of tyrosinase into the active conformation (Petrescu et al., 1997), completely inhibited melanin formation unless exogenous L-DOPA was added to bypass the requirement for tyrosinase (Fig. 2 B). These results suggested that tyrosinase was not expressed or not active in GM95 cells. To discern between these possibilities, we analyzed tyrosinase expression in GM95 and MEB4 cells by Western blot. As shown in Fig. 2 C, GM95 cells expressed the same amount of tyrosinase (70–75 kD apparent MW) as MEB4 cells. Importantly, this figure also documented tyrosinase-related protein (TRP)-1-dependent L-DOPA oxidase activity (85–90 kD apparent MW) (see Fig. 6; Jimenez-Cervantes et al., 1993a). Thus, despite its expression and *in vitro* activity in glycosphingolipid-deficient GM95 cells, tyrosinase failed to synthesize L-DOPA.

Tyrosinase is not localized in melanosomes in GM95 cells

Comparison of similar regions in MEB4 and GM95 cells by EM on plastic sections (Fig. 3, A and C) showed that although GM95 cells contain many endosome/melanosome-like vacuoles, the characteristic dark melanin pigmentation

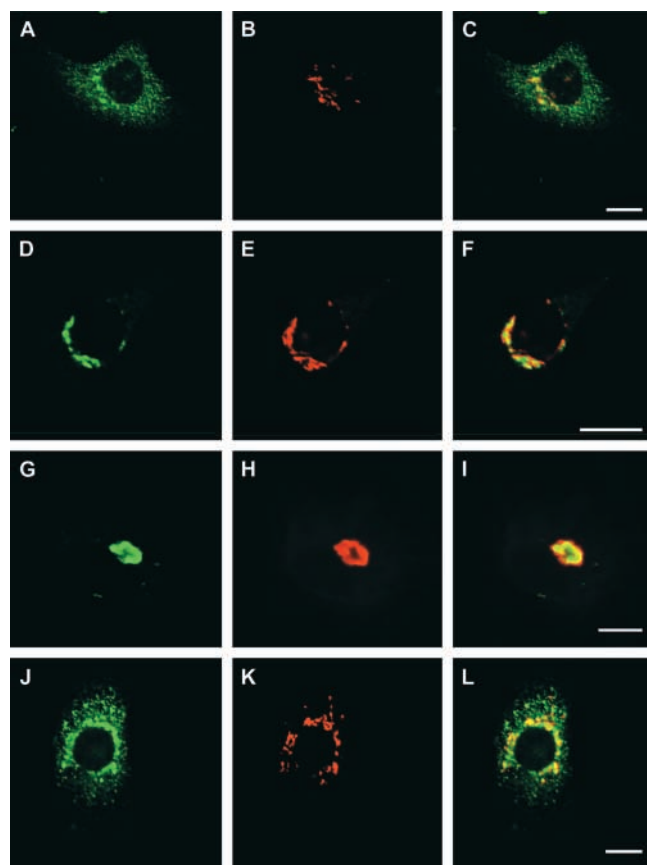


Figure 4. Localization of tyrosinase. MEB4 (A–C), GM95-mock (D–F), and GM95-CGlcT-KKVK (J–L) cells were fixed and labeled with rabbit antityrosinase antiserum anti-pep7 (A, D, G, and J) and, to mark the Golgi complex, with mouse anti-CTR433 antibody (B, E, and K) or anti-myc-sialyltransferase (H). Cells were counterstained with FITC-labeled goat anti-rabbit (A, D, G, and J) and Texas red-labeled goat anti-mouse (B, E, H, and K) antisera. Coverslips were analyzed by confocal fluorescence microscopy. Areas of overlapping distributions in the same optical section appear as yellow in the merged images (C, F, I, and L). Every pixel had a value between 1 and 255. Bars, 10 μ m.

was absent. To investigate the localization of the downstream reactions in melanin biosynthesis, we preincubated the cells with L-DOPA. Whereas MEB4 cells displayed an increase in the number of pigmented melanosomes (Fig. 3 B), L-DOPA treatment of GM95 cells caused pigment deposition in the vacuolar compartments (Fig. 3 D). Notably, pigmentation never occurred in the Golgi complex. These morphological experiments suggested that melanosomal proteins involved in later steps of pigmentation were still transported to post-Golgi vacuoles in GM95 cells.

Tyrosinase is inactive outside melanosomes (Beermann et al., 1995). To determine whether tyrosinase was not localized to melanosomes in GM95 cells, we investigated its distribution by double label immunofluorescence microscopy using antibodies against tyrosinase, the medial-Golgi marker CTR433 (Jasmin et al., 1989), and the myc-tagged trans-Golgi marker sialyltransferase (Munro, 1991). Tyrosinase was localized predominantly to punctate cytoplasmic structures in MEB4 cells. In addition, we found some tyrosinase in the perinuclear region as shown in Fig. 4, A–C. This label-

ing pattern is typical for melanosomes as illustrated by electron micrographs of MEB4 cells (Fig. 3). In contrast, antibodies against tyrosinase labeled few if any peripheral structures in GM95 cells, and labeling was limited essentially to the perinuclear region (Fig. 4, D and G). The distribution of tyrosinase in the GM95 cells was closely similar but not identical to that of the medial- and trans-Golgi markers (Fig. 4, D–I), suggesting that tyrosinase was located either in the TGN or in the cis-Golgi and ER-Golgi intermediate compartment. Since pigmentation was restored in the GM95 transfectant expressing CGlcT-KKVK, we also analyzed the localization of tyrosinase in this cell line. Consistent with the ability of this transfectant to synthesize L-DOPA and pigment, we found a large fraction of tyrosinase localized to peripheral structures outside the Golgi area (Fig. 4, J–L) as in MEB4 cells. The fraction of tyrosinase in the periphery was lower in GM95-CGlcT-KKVK than in MEB4, which fits their lower level of glycosphingolipids and pigmentation (Fig. 1). In GM95 cells, tyrosinase is associated exclusively with the Golgi complex and unable to synthesize L-DOPA.

Extension of the tyrosinase transmembrane domain restores pigmentation in GM95 cells

The localization of tyrosinase in the Golgi complex of GM95 cells suggested that the sorting information in the protein needed for transport out of the Golgi complex is no longer recognized and that a secondary signal is responsible for Golgi arrest. One typical Golgi retention signal is a short transmembrane domain of ~ 17 amino acids (Munro, 1995), and indeed mouse tyrosinase has a predicted (Hofmann and Stoffel, 1993) transmembrane domain of 17 amino acids. To investigate whether this domain was responsible for its retention in the Golgi complex, we generated stable GM95 transfectants expressing tyrosinase with an extended transmembrane domain, tyrosinase-TM6. Even GM95 cells with a low (twofold) overexpression of tyrosinase-TM6 produced L-DOPA very efficiently (Table I) and regained the ability to produce pigment as documented by the black cell pellet in Fig. 5 A. Consistent with this observation, tyrosinase-TM6 localized to vacuolar structures in the cytoplasm (Fig. 5 B). To rule out the possibility that overexpression caused saturation of the sorting machinery in the Golgi complex and allowed tyrosinase to escape, we generated a stable GM95 transfectant with a similar expression level of wild-type tyrosinase. These cells remained white, and the ectopically expressed tyrosinase was localized to the Golgi complex (Fig. 5, A and B) like endogenous tyrosinase (Fig. 4 D). Tyrosinase distribution outside the Golgi area and a minimal amount of pigmentation became apparent upon sixfold overexpression. The results in the tyrosinase-TM6 and tyrosinase GM95 transfectants showed that the pigmentation defect in cells without glycolipids is due solely to mislocalization of tyrosinase and that its arrest in the Golgi complex is due to a cryptic Golgi retention signal.

The extended transmembrane domain could have restored transport of tyrosinase-TM6 to peripheral vacuoles by targeting the protein into a direct pathway from the Golgi complex to melanosomes. Alternatively, tyrosinase-TM6 might be transported to the plasma membrane and internalized by

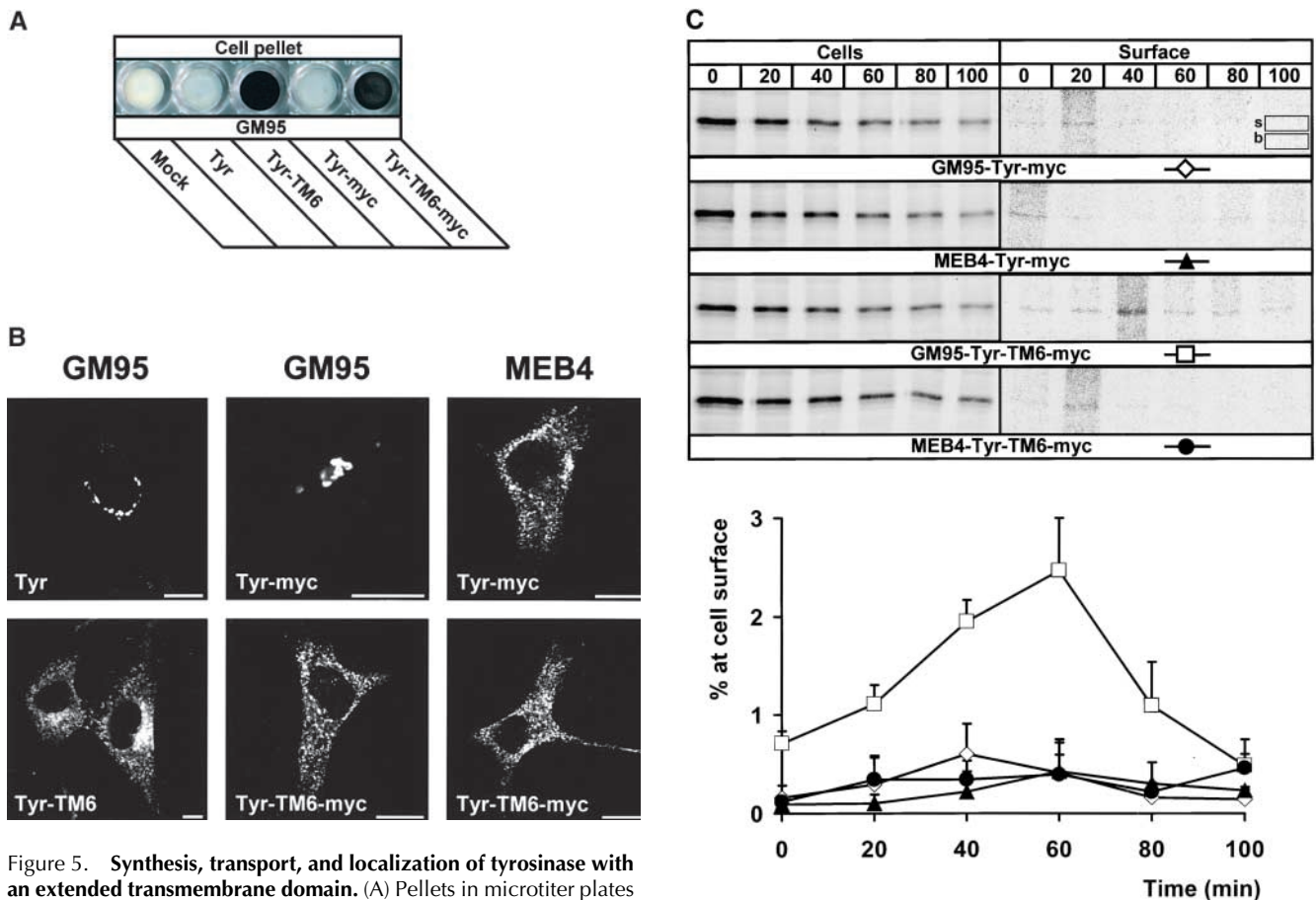


Figure 5. Synthesis, transport, and localization of tyrosinase with an extended transmembrane domain. (A) Pellets in microtiter plates of GM95 cells transfected with empty vector (Mock), tyrosinase (Tyr), Tyr-TM6, Tyr-myc, and Tyr-TM6-myc. GM95-Tyr and GM95-Tyr-TM6 cells overexpressed tyrosinase two- to threefold as assayed by Western blotting. (B) The distribution of tyrosinase was analyzed by confocal fluorescence microscopy using the anti-pep7 antibody or the anti-myc antibody and an FITC-labeled secondary antibody. Anti-pep7 labels both endogenous tyrosinase in the Golgi of these cells (compare with Fig. 4) and transfected tyrosinase. (C) GM95 and MEB4 cells transfected with tyrosinase-myc or tyrosinase-TM6-myc were pulse labeled for 60 min with Tran-³⁵S-label, chased for the indicated time (min), and biotinylated on ice. Tyrosinase was immunoprecipitated from detergent lysates with the anti-myc antibody. Immunoprecipitated protein was eluted from the beads, and part was analyzed by SDS-PAGE and fluorography (Cells). Biotinylated tyrosinase was immunoprecipitated from the remainder using streptavidin-agarose beads and analyzed by SDS-PAGE and phosphorimaging (Surface). Tyrosinase (~70 kD) at the surface was quantified by subtracting a blank value (b) from the signal (s) in each lane and was divided by the signal of the cells at $t = 0$ in a phosphorimage obtained under the same conditions (unpublished data) and expressed as percent of total at $t = 0$. Tyrosinase is degraded rapidly. This occurs in the ER and is due to inefficient folding (Branza-Nichita et al., 1999). Data are the mean of two independent experiments. Bars represent range.

endocytosis. To discriminate between these possibilities, MEB4 and GM95 cells were stably transfected with tyrosinase-myc and tyrosinase-TM6-myc. We used myc-tagged constructs for these experiments, since anti-pep7 antibody did not efficiently immunoprecipitate tyrosinase. The epitope tag did not affect the steady-state distributions of tyrosinase and tyrosinase-TM6 (Fig. 5, A and B). The appearance of newly synthesized proteins on the cell surface was then determined in pulse-chase experiments and cell surface biotinylation. Little if any tyrosinase-myc was found on the plasma membrane of GM95 and MEB4 cells (Fig. 5 C) at any chase time, suggesting that it was transported from the Golgi complex to the melanosome via a direct intracellular pathway. In contrast, we found that tyrosinase-TM6-myc was observed consistently on the surface of GM95 cells and the amount significantly increased during the chase (Fig. 5 C). Thus, extension of the tyrosinase transmembrane do-

main enhanced transport of tyrosinase-TM6 from the Golgi complex to the plasma membrane in GM95 cells. This effect was specific for GM95 cells since it did not occur in MEB4 cells transfected with the same construct. This suggested that the signal responsible for tyrosinase-TM6-myc transport in the direct pathway is dominant over the plasma membrane route. In GM95 cells, the signal for transport to the melanosome is either nonfunctional or the pathway no longer exists.

TRP-1 reaches peripheral vacuoles in GM95 cells via the cell surface

Addition of L-DOPA to GM95 cells resulted in pigment synthesis in peripheral vacuoles (Figs. 2 and 3), implying that a DOPA-oxidase activity other than tyrosinase must be present in these organelles. Because this activity is due typically to the melanosomal protein TRP-1 (Fig. 2 C), we investigated the distribution of endogenous TRP-1 in GM95

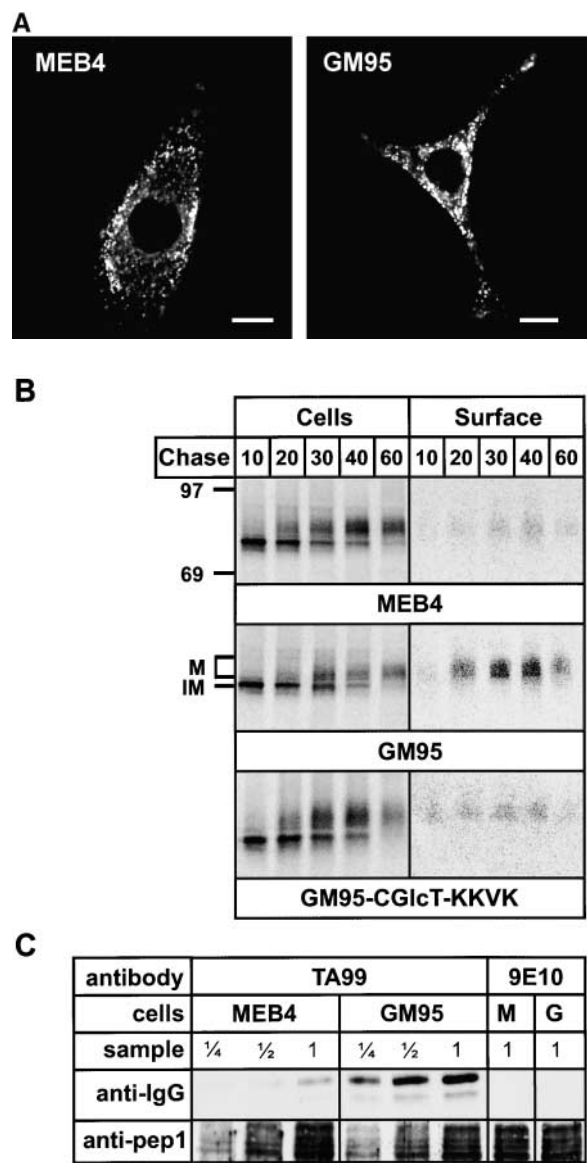


Figure 6. Localization and transport of TRP-1. (A) MEB4 and GM95 cells were fixed, permeabilized, and incubated with the anti-pep1 antibody against TRP-1, counterstained with FITC-labeled secondary antibody, and viewed by confocal fluorescence microscopy. (B) MEB4, GM95, and GM95-CGlcT-KKVK cells were pulse labeled for 15 min, chased for the indicated time (min), and biotinylated like tyrosinase, described in the legend to Fig. 5, but using the TA99 antibody to immunoprecipitate TRP-1. Data (phosphorimages) are representative of three experiments. IM, immature core-glycosylated form; M, mature complex-glycosylated forms. (C) Cells were incubated with TA99 antibody against the exoplasmic domain of TRP-1 or with the control mouse anti-myc mAb 9E10 for 3 h at 37°C. After washing, internalized antibody was visualized by SDS-PAGE and Western blotting using anti-IgG coupled to HRP. Reactivity of 9E10 with the anti-IgG was controlled by a Western blot of myc-tagged sialyltransferase (unpublished data). To allow a quantitative comparison, different amounts of each lysate were loaded on the gel, and as an internal control the total amount of TRP-1 in the samples (present as mature and immature forms) was measured by Western blotting using anti-pep1. Bars, 10 μ m.

cells. TRP-1 was concentrated in punctate structures throughout the cytoplasm (Fig. 6 A). TRP-1 is transported from the Golgi complex to melanosomes via the direct path-

way (Vijayasaradhi et al., 1995; Jimbow et al., 1997; Raposo et al., 2001). To investigate whether TRP-1 still followed this route in GM95 cells, we also analyzed its transport in a pulse-chase and cell surface biotinylation experiment (Fig. 6 B). The rate of synthesis and glycosylation of TRP-1 was identical in MEB4 and GM95 cells, indicating that biosynthetic transport through the ER and Golgi complex was not affected in GM95 cells. Also the rate of degradation, ~20% in 60 min, was identical in the two cell lines. In contrast, the fraction of newly synthesized TRP-1 present on the cell surface at various time points during 1 h of chase increased six-fold in GM95 cells compared with MEB4 cells. The fraction of TRP-1 on the plasma membrane of the GM95 transfectant expressing CGlcT-KKVK was reduced to similar levels as in MEB4 cells (Fig. 6 B).

Enhanced delivery of TRP-1 to the surface of GM95 cells suggested that TRP-1 is not transported directly from the Golgi complex to melanosomes but instead reached the melanosome via endocytosis from the plasma membrane. We tested this idea in an independent experiment in which MEB4 and GM95 cells were incubated at 37°C with TA99, an antibody against the exoplasmic portion of TRP-1. The relative amount of endocytosed antibody molecules was then determined by Western blot of cell lysates. In three experiments, four to six times more TA99 was taken up by the GM95 cells, whereas an irrelevant antibody was not internalized by GM95 nor by MEB4 cells (Fig. 6 C). These data show enhanced transport of TRP-1 via the cell surface to melanosomes in the absence of glycosphingolipids. Immunofluorescence microscopy on parallel dishes with FITC-labeled goat anti-mouse antibody confirmed that the bulk of internalized antibody resided in peripheral endocytic compartments (unpublished data). The predicted transmembrane domain of TRP-1 comprises 24 amino acids (Hofmann and Stoffel, 1993). As with tyrosinase-TM6-myc, the long transmembrane domain appears to function as a plasma membrane signal only in the absence of glycosphingolipids.

Transport of lysosomal enzymes from the Golgi complex to lysosomes is unchanged in GM95 cells
Melanosomes are considered to be specialized endosomes/lysosomes. Besides the pathway from the Golgi complex to the melanosome that is apparently disrupted in GM95 cells, one other direct pathway is known from the Golgi to endosomes. The best documented examples of cargo molecules transported via this pathway are the two mannose 6-phosphate receptors (MPRs) (Puertollano et al., 2001; Zhu et al., 2001) that mediate transport of most soluble lysosomal enzymes to endosomes (Kornfeld, 1992). The small fraction of lysosomal enzymes that fails to bind to the MPRs is secreted and partially recaptured after binding to MPRs on the cell surface. MPR sorting in the pathway depends on the adaptor complex AP-1. Fibroblasts with a nonfunctional AP-1 missort cathepsin D resulting in a threefold increase in the release of cathepsin D precursor forms into the medium (Meyer et al., 2000). Thus, a defect in the direct transport pathway for the MPRs should cause an increased release of lysosomal enzymes.
We investigated whether the MPR pathway was affected in GM95 cells by assaying secretion of two lysosomal hydro-

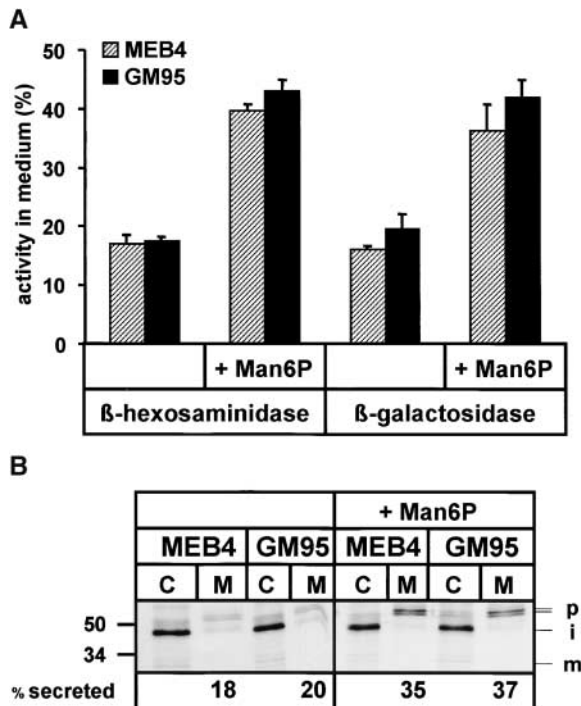


Figure 7. **Secretion of lysosomal enzymes.** (A) MEB4 cells and GM95 cells were cultured for 36 h in the presence or absence of 5 mM Man6P, and the activities of β -hexosaminidase and β -galactosidase were determined in the media and in the cells. Tissue culture medium and lysis buffer were used as background. Signals were 30–40 times over background. Data are the mean of two experiments ($n = 4$). (B) MEB4 and GM95 cells were pulse labeled for 60 min and chased in the presence or absence of 5 mM Man6P for 3.5 h. Cathepsin D was immunoprecipitated from the media and detergent lysates with the anti-cathepsin D antibody. Immunoprecipitated proteins were analyzed by SDS-PAGE and phosphorimaging. The precursors (p), intermediate (i), and mature (m) forms of cathepsin D are indicated. Numbers indicate the mean percentage of cathepsin D secreted into the medium of three independent experiments ($SD < 6\%$).

lases that are known to bind to MPRs (Ludwig et al., 1994). About 20% of β -hexosaminidase and β -galactosidase was secreted in 36 h (Fig. 7 A). In the presence of mannose 6-phosphate (Man6P), secretion increased twofold in both the MEB4 and GM95 cells. In addition, we determined transport (and maturation) of newly synthesized cathepsin D to lysosomes and its secretion into the medium. In the media of both cell lines, two immature forms of cathepsin D (51 and 53 kD) were detected, probably precursors (p) with different *N*-glycans (Fig. 7 B). Both cell lysates contained a small amount of the precursors. The main detected species was the 44 kD intermediate (Fig. 7 B, i), which results from a first cleavage in endosomes, whereas very little mature cathepsin D (Fig. 7 B, m; 31 kD) was detected. The amount of cathepsin D secreted as the percentage of the total forms was the same in both cell lines (18% for MEB4 and 20% for GM95). In the presence of 5 mM Man6P, a twofold increase in the secretion of the 51 and 53 kD precursors was observed (35% for MEB4 and 37% for GM95), showing that in both cell lines half of the secreted cathepsin D molecules are recaptured by MPR-mediated endocytosis. The unchanged secretion of three hydrolases and the identical effect of Man6P in GM95 and MEB4 cells showed that the direct

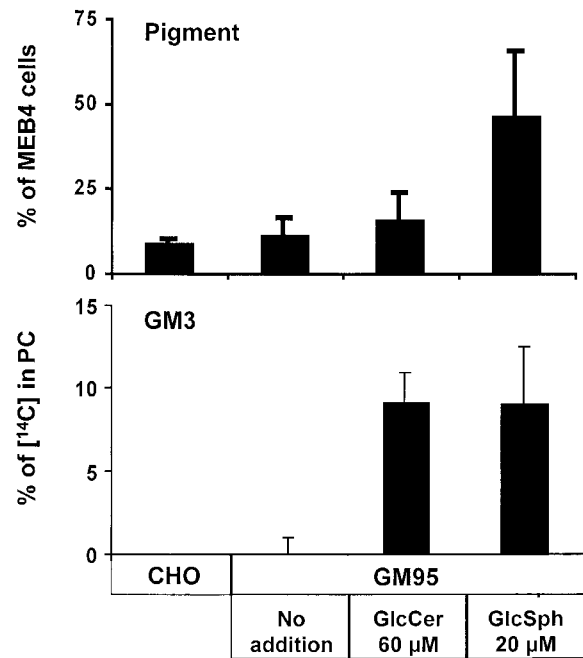


Figure 8. **Exogenous GlcSph but not GlcCer induces pigmentation in GM95 cells.** COS7, GM95, and MEB4 cells were grown in high glucose DME containing 2% FCS and 1 mM tyrosine. GM95 cells were grown in the presence or absence of GlcCer or GlcSph. (Top) After 3 d, the melanin content of cell pellets was measured colorimetrically (A475 nm/protein). (Bottom) After addition of GlcCer or GlcSph, parallel dishes of cells were labeled overnight with D-[14 C]galactose. Lipids were extracted, separated by two-dimensional TLC, and the GM3 spot was visualized and quantitated using phosphorimaging. No LacCer spot was observed. [14 C]GM3 was expressed as a percentage of the radioactivity in the phosphatidylcholine (PC) spot as an internal standard. Data are means of triplicate experiments \pm SD.

MPR transport pathway in GM95 cells was not affected by the absence of glycolipids.

Exogenous glucosylsphingosine restores pigmentation

Transfection of GM95 cells with CGlcT or CGalT restored pigmentation. Because these enzymes transfer glucose and galactose from UDP-glucose and -galactose, respectively, it could be argued that pigmentation either required removal of ceramide or alternatively the production of glycosphingolipids. To test this, GM95-CGlcT or GM95-CGalT cells were incubated with exogenous ceramide for 3 d. If the block in transport of tyrosinase in GM95 cells would be caused by increased ceramide levels, addition of exogenous ceramide might inhibit tyrosinase transport and pigmentation in the GM95-CGlcT or -CGalT cells. However, the opposite was observed. When GM95-CGlcT or -CGalT cells were incubated with 20 μ M ceramide, pigmentation increased 1.7- and 1.5-fold, respectively. Furthermore, ceramide addition caused a 5.3-fold increase in the synthesis of glycosphingolipids in GM95-CGlcT cells and 1.6-fold in GM95-CGalT cells, whereas sphingomyelin was increased 1.3-fold in both cell types. In GM95 cells, ceramide addition enhanced sphingomyelin synthesis but not pigmentation. Thus, pigmentation correlated with the level of glycosphingolipids and not with that of ceramide or sphingomyelin.

Exogenous GlcCer did not restore pigmentation in GM95 cells. However, when we used an alternative method to raise the intracellular level of GlcCer, incubation with glucosylsphingosine (GlcSph), which is intracellularly converted to GlcCer, pigmentation occurred to a level of 50% that of MEB4 cells (Fig. 8). GlcSph restored tyrosinase transport, resulting in significant peripheral staining of Tyr-myc after 8 h and full redistribution after 1 d. Both GlcCer and GlcSph entered the GM95 cells, as they were converted to GM3, that could be radiolabeled by simultaneous addition of [14 C]galactose (Fig. 8) or [14 C]*N*-acetylmannosamine, a precursor for sialic acid (unpublished data). The induction of pigmentation was not due to detergent activity of GlcSph because the same concentration of galactosylsphingosine had no effect nor could it be achieved by just any glucose in a β 1-linkage to a lipidic tail, since 10–100 μ M octyl- β -D-glucoside or 50 μ M octylglucoside and 50 μ M GlcCer did not affect pigmentation. The data suggest that transport of tyrosinase from the Golgi depends on GlcSph itself or a pool of GlcCer situated at a location inaccessible to exogenous GlcCer. Since exogenous GlcCer reached the Golgi lumen, as evidenced by its conversion to GM3, the active pool of GlcCer may reside on the cytosolic surface of the TGN.

Discussion

Mislocalization of tyrosinase is responsible for the absence of pigment from GM95 cells

Here, we report the loss of pigmentation in the melanoma mutant cell line GM95 and the fact that this is specifically due to a block in the first step in melanin synthesis, the conversion of tyrosine to L-DOPA by tyrosinase. Tyrosinase is not required for the subsequent reactions, since addition of L-DOPA to melanoma cells in which tyrosinase had been inactivated by NB-DNJ allowed these white cells to form pigment and turn black (Fig. 2 B; Negroiu et al., 1999; NB-DNJ did not inhibit tyrosinase by blocking GlcCer synthesis as the inactive tyrosinase still made it to the melanosomes; unpublished data). In addition, GM95 cells converted exogenous L-DOPA to melanin in structures (Fig. 3) that were essentially devoid of tyrosinase (Fig. 4; exogenous L-DOPA did not induce redistribution of tyrosinase to melanosomes by immunofluorescence; unpublished data). In vivo, tyrosinase activity may be affected in several ways. First, its expression level may vary due to changes in synthesis (Slominski et al., 1988; Brown et al., 1998) or turnover (Martínez-Esparza et al., 1997). Second, tyrosinase may be inactive due to a mutation of the active site (Oetting and King, 1999). Third, various defects in transport of tyrosinase to the melanosome affect pigmentation. In the platinum mouse, a mutated tyrosinase with a truncated cytoplasmic tail bypasses the melanosomes, which results in severe oculocutaneous albinism (Beermann et al., 1995). In contrast, in amelanotic human melanoma cells wild-type tyrosinase is retained more efficiently in the ER and degraded by the proteasome (Halaban et al., 2001). In the present study, a defect in glycosphingolipid synthesis caused retention of tyrosinase in the Golgi (Fig. 4) and abrogated pigmentation (Fig. 1), whereas this tyrosinase was fully active in vitro. We conclude that tyrosi-

nase is unable to perform its biochemical function in the Golgi lumen. One potential difference between the Golgi and melanosome is the exclusive presence in the melanosome of a transporter that allows tyrosine to enter the lumen where the active center of tyrosinase is located. Selective relocalization of tyrosinase to the melanosome by a mutation in its transmembrane domain restored pigmentation (Fig. 5). This shows that tyrosinase mislocalization itself was responsible for the pigmentation loss in GM95 cells.

Melanosomal protein sorting in the absence of glycosphingolipids

A dileucine containing motif in the cytosolic tail is required for proper targeting of several melanosomal membrane proteins including tyrosinase and TRP-1 (Vijayasarathe et al., 1995; Calvo et al., 1999; Simmen et al., 1999). The tyrosinase dileucine motif interacts with the AP-3 adaptor complex (Höning et al., 1998) and not with AP-1, a distinct adaptor involved in sorting endosomal membrane proteins toward endosomes (Kirchhausen, 1999). The significance of AP-3 in sorting tyrosinase to the melanosome is probably best illustrated by the pearl mouse, where a mutation in AP-3's β 3A subunit causes hypopigmentation (Feng et al., 1999). Down-regulation of AP-3 levels with antisense oligonucleotides (Le Borgne et al., 1998) or in Hermansky-Pudlak syndrome patients lacking the β 3A subunit (Dell'Angelica et al., 1999) re-directs AP-3-dependent lysosomal membrane proteins to the cell surface. We found a comparable effect for TRP-1 and tyrosinase-TM6 in glycosphingolipid-deficient cells, suggesting that glycosphingolipids are essential for protein sorting in the direct Golgi to melanosome pathway or that this pathway no longer operates. The indirect pathway may also be responsible for the continued TRP-1 transport observed in melanocytes lacking functional AP-3 (Huizing et al., 2001). The requirement for glycosphingolipids in GM95 cells was specific for the Golgi to melanosome pathway, since the MPR transport pathway from the Golgi to endosomes was not affected by their absence (Fig. 7).

The compartments reached by TRP-1 and tyrosinase-TM6 in the GM95 cells (Figs. 5 and 6) are indistinguishable from melanosomes in MEB4 cells at the light microscopical level and probably the same organelles turned electron dense upon addition of exogenous L-DOPA (Fig. 3). However, in contrast to the MEB4 melanosomes the GM95 compartments sometimes carry tubular extensions (Fig. 3 D). In addition, our preliminary evidence from ultrastructural immunolocalization experiments with several marker proteins suggests that melanosomes are distinct from lysosomes in MEB4 cells and melanocytes (Raposo et al., 2001) but are no longer separate compartments in GM95 cells. This suggests that the proper organization of the secretory/endocytic system depends on an active direct Golgi to melanosome pathway.

How do glycosphingolipids enable tyrosinase transport?

The primary defect in GM95 cells is the lack of glycosphingolipid synthesis due to the absence of CGlT activity. Only a very low signal for CGlT mRNA was found in these cells by Northern blot analysis (Ichikawa et al., 1994). Transfec-

tion with either CGlcT or CGalT restored transport and sorting of tyrosinase and TRP-1, DOPA synthesis, and pigmentation (Figs. 1, 4, and 6 and Table I). In addition, exogenous GlcSph stimulated pigmentation (Fig. 8). We conclude that CGlcT and CGalT restored tyrosinase transport by producing glycosphingolipids rather than by removing inhibitory ceramide (Rosenwald and Pagano, 1993). Indeed, exogenous ceramide did not inhibit but increased pigmentation in MEB4, GM95-CGlcT, and GM95-CGalT cells but not GM95 cells (unpublished data).

Glycosphingolipid and cholesterol-enriched domains in the luminal leaflet of the Golgi are thought to play an essential role in the sorting of proteins toward the apical plasma membrane domain of epithelial cells (Simons and van Meer, 1988; Sprong et al., 2001). The fact that exogenous GlcSph and GlcCer yielded the same concentration of GM3 but only GlcSph restored pigmentation (Fig. 8) implies that the presence of glycolipids in the luminal leaflet of the Golgi is not crucial for restoring tyrosinase transport from the Golgi. The observation that a GPI protein is detergent insoluble and transported to the plasma membrane suggests the persistence of a typical raft pathway in GM95 cells (Ostermeyer et al., 1999). The observations that TRP-1 and tyrosinase with an extended transmembrane domain but not tyrosinase escape the Golgi toward the plasma membrane support the idea that the vesicles toward the plasma membrane have thicker membranes as can be expected from sphingolipid/cholesterol rafts in the TGN (Munro, 1995; Sprong et al., 2001). In addition, these data suggest that tyrosinase accumulated in the TGN rather than the cis Golgi.

There are various scenarios for how GlcSph but not GlcCer restores transport. (a) GlcSph could be involved directly in the budding step. Potentially, it is the production of GlcSph and not of GlcCer that is required for tyrosinase transport. Due to the low concentration of GlcSph in cells, it would be more likely involved in signaling than in a structural function. (b) GlcCer may be required on the cytosolic surface of the Golgi. GlcCer is synthesized by CGlcT on the cytosolic surface of the Golgi, and it is most likely that acylation of GlcSph occurs on the cytosolic surface. In contrast, exogenous GlcCer may be limited to the exoplasmic leaflet even during endocytic recycling (van Genderen and van Meer, 1995; van IJzendoorn and Hoekstra, 1998) (for different data see Martin and Pagano, 1994). GlcCer may form domains on the cytosolic surface of the TGN that would then be involved in melanosomal vesicle budding. However, tyrosinase is completely soluble in 0.5% Triton X-100 and in 0.5% Lubrol WX at 4°C in both MEB4 cells and in GM95 cells (unpublished data). Alternatively, GlcCer on the cytosolic surface could recruit coat proteins from the cytosol. One candidate is the cytosolic glycolipid transfer protein (Mattjus et al., 2000).

Synthesis of GalCer also restored tyrosinase transport (Fig. 1). Although GalCer is synthesized in the luminal leaflet of the ER membrane, experiments with short chain GalCer have demonstrated that it has access to the same locations as GlcCer (Burger et al., 1996). GalCer and GlcCer have similar self-aggregation properties, and both GlcCer and GalCer can bind the glycolipid transfer protein (Mattjus et al., 2000).

We observed recently in fibroblasts that half of newly synthesized GlcCer is transported to the plasma membrane on

the cytosolic surface of transport vesicles. Subsequently, it is removed from the cytosolic side by the multidrug transporter MDR1 P-glycoprotein. An attractive scenario would be that GlcCer is involved in the recruitment of the coat protein complex in the TGN. After vesicle budding, GlcCer as a co-factor would be removed by MDR1 and reattachment of the coat complex prevented. Such a mechanism may be similar to the regulation of AP-1 and AP-2 activities by phosphoinositides (Kirchhausen, 1999).

The unexpected observation that glycosphingolipids are essential for a protein-sorting step in the Golgi complex but not for transport toward the plasma membrane shines a new light on the roles of glycosphingolipids in vesicular traffic. Our present findings indicate that some pigmentation defects notably in the class of the Hermansky-Pudlak syndrome may find their origin in aspects of glycosphingolipid metabolism. Such studies are now underway.

Materials and methods

Reagents

Bovine ceramide was obtained from Matreya, and C₆-NBD-ceramide was purchased from Molecular Probes. GlcCer and GlcSph were from Sigma-Aldrich. NB-DNJ was a gift from F. Platt (University of Oxford, Oxford, UK) (Petrescu et al., 1997). M. Bornens (Institute Curie, Paris, France) provided us with mouse mAb against CTR433. Rabbit antisera against the cytoplasmic tail of tyrosinase (anti-pep7) and TRP-1 (anti-pep1) were gifts from V. Hearing (National Institutes of Health, Bethesda, MD) (Jimenez et al., 1988). The rabbit polyclonal A-14 against the c-myc epitope tag was from Santa Cruz Biotechnology, Inc., and the mouse mAb 9E10 has been described (Evan et al., 1985). The mouse mAb TA99 was provided by K. Lloyd (Memorial Sloan Ketterin Cancer Center, New York, NY) (Thomson et al., 1985). Rabbit anti-cathepsin D was a gift from K. von Figura (Georg-August University, Göttingen, Germany) (Meyer et al., 2000). Fluorescently labeled secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc., and HRP-conjugated goat anti-mouse IgG was from Dako. Mouse tyrosinase cDNA was provided by F. Beermann (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland) (Simmen et al., 1999), and myc-tagged sialyltransferase cDNA was provided by S. Munro (Medical Research Council, Cambridge, UK) (Munro, 1991).

Plasmid construction

CGlcT and CGlcT-KKVK containing an ER retrieval signal at the COOH terminus were amplified in PCR reactions using CGlcT-pcDNA3 as template (Sprong et al., 1998) and ligated in pCB7 (Hansen and Casanova, 1994). cDNAs encoding CGalT (van der Bijl et al., 1996b), tyrosinase (Simmen et al., 1999), and myc-tagged sialyltransferase (Munro, 1991) were released from the original plasmids and inserted in pCB7. The putative transmembrane domain of tyrosinase was extended with the hexapeptide VLALVA to generate tyrosinase-TM6 by ligating the oligonucleotide 5'-pGTACTAGCACTAGTTGCA-3' in the PstI site. A double myc tag was appended at the COOH terminus of tyrosinase and tyrosinase-TM6 in sequential PCR reactions and ligated in pCB7. Primers used in PCR reactions are available on request. All constructs made by PCR were confirmed by sequencing both strands.

Cell culture and transfection

CHO, GM95, and MEB4 cells were maintained and transfected as described (Sprong et al., 1998), whereby GM95 and MEB4 cells were grown in DME containing 10% FCS. Stable transfectants were selected in the presence of 200 U/ml hygromycin B. Expression was analyzed by measuring enzyme activity, Western blot, or immunofluorescence microscopy. For pigmentation experiments, 1 mM L-tyrosine was included in the medium at the time of plating the cells.

Morphological methods

Subconfluent cells were incubated for 3 h at 37°C with/without 1 mM L-DOPA. The cells were fixed overnight at 4°C with 2% paraformaldehyde and 2.5% glutaraldehyde, postfixed with 1% OsO₄, scraped, and embedded in epon. Ultrathin sections were stained with 2% uranylacetate in distilled water for 45 min at 63°C. Immunofluorescence microscopy was per-

formed as described (Sprong et al., 1998) with the modification that we used PBS containing 10% goat serum, 0.5% BSA, and 0.1% saponin as blocking buffer.

Melanin and L-DOPA content

Subconfluent cells on 10-cm dishes at 3–5 d after seeding were incubated for 3 h at 37°C with/without 1 mM L-DOPA. The cells were washed three times and gently scraped in ice-cold PBS. The protein content was determined in a fraction of the cells using the BCA assay (Pierce Chemical Co.). The remaining cells were transferred to a 96-well microtiter plate and pelleted at 1,000 g. To solubilize melanin, cell pellets were resuspended in 1 ml 1 M NaOH, vortexed vigorously, and boiled for 30 min (Friedmann and Gilchrist, 1987). Pigmentation was measured as A_{475} /mg protein. L-DOPA in homogenates and culture media was determined by reverse-phase HPLC on a LC18 DB column (Supelco) using 0.1 M TCA adjusted with sodium acetate to pH 3.2 as a mobile phase and electrochemical detection as described (Westerink et al., 1982).

Metabolic labeling of lipids

Subconfluent cells on 3-cm dishes were incubated with 1.5 ml culture medium containing D-erythro-[3- 3 H]sphingosine (0.65 TBq/mmol; 67 kBq/ml; NEN Dupont), D-[1- 14 C]galactose (37 kBq/ml), or [1- 14 C]acetic acid (both 1.8 GBq/mmol; 37 kBq/ml; Amersham Pharmacia Biotech) in the presence or absence of drugs or lipid analogs for 16–48 h. Cells were washed three times with ice-cold PBS. Lipids were extracted and separated by one-dimensional TLC in chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5 vol/vol) or by two-dimensional chromatography in chloroform/methanol/25% NH_4OH /water (65:35:4:4 vol/vol) and chloroform/methanol/ CaCl_2 (65:30:8 vol/vol). Radiolabeled spots were visualized by fluorography using X-ray films, scraped, and quantitated as described (van der Bijl et al., 1996a).

Pulse–chase and cell surface biotinylation

Confluent cells on 3-cm dishes were incubated for 30 min at 37°C with methionine- and cysteine-free DME containing 20 mM Hepes, pH 7.4 (pulse medium), and labeled with 18 MBq/ml Tran- ^{35}S -label (ICN Biomedicals) for 15 or 60 min at 37°C. Cells were chased in growth medium containing 5 mM methionine, 5 mM cysteine, and 20 mM Hepes, pH 7.4, at 37°C. Arrival of newly synthesized proteins at the plasma membrane was assayed by cell surface biotinylation (Hunziker et al., 1991). Briefly, the cells were incubated twice with 0.5 mg/ml sulfo-NHS-SS-biotin in PBS for 20 min and quenched with 10 mM glycine. Cells were lysed in PBS, 10 mM glycine, 0.5% vol/vol TX-100, 1 mM EDTA, pH 8.0, 1 mM PMSF, and 1 $\mu\text{g}/\text{ml}$ of the protease inhibitors aprotinin, chymostatin, leupeptin, and pepstatin A. TRP-1, tyrosinase-myc, and tyrosinase-TM6-myc were then immunoprecipitated from precleared detergent lysates as described (Sprong et al., 1998). Immune complexes were eluted in 150 mM NaCl, 2 mM EDTA, 100 mM Tris-HCl, pH 8.3, 0.5% wt/vol SDS, 1 mM PMSF, and 1 $\mu\text{g}/\text{ml}$ protease inhibitors for 15 min at 37°C, and 20% was saved to measure the amount of metabolically labeled protein. The remainder was diluted 30-fold with 150 mM NaCl, 2 mM EDTA, 100 mM Tris-HCl, pH 8.3, 0.1% wt/vol SDS, 0.5% wt/vol NP-40, 0.5% wt/vol sodiumdeoxycholate, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ protease inhibitors, and incubated for 1 h with streptavidin beads. Beads were washed four times with dilution buffer, and bound protein was resolved by SDS-PAGE. Quantitations were done by phosphorimaging using Imagequant software.

Antibody internalization

Cells in 3-cm dishes were incubated for 3 h at 37°C with 50 $\mu\text{g}/\text{ml}$ anti-TRP-1 antibody TA99 or control antibody 9E10 in medium containing 20 $\mu\text{g}/\text{ml}$ leupeptin. Cells were washed five times with ice-cold PBS and lysed in reducing Laemmli sample buffer. Equal amounts of protein were analyzed by SDS-PAGE, and internalized antibody was detected by Western blotting as before (Sprong et al., 1998). Reactivity of 9E10 with the anti-IgG was controlled by a Western blot of myc-tagged sialyltransferase. As an internal control, TRP-1 was detected using the rabbit antibody anti-pep1.

Miscellaneous methods

Fractionation of GM95 cells and separation of ER and Golgi membranes on sucrose gradients was performed as described (Burger et al., 1996). β -Galactosidase and β -hexosaminidase activity in cell lysates and media were analyzed according to Galjaard (1980) and Aerts et al. (1991). L-DOPA oxidase activity of tyrosinase and TRP-1 was detected by zymography of 10% SDS-PAA gels run in nonreducing Laemmli sample buffer (Jimenez-Cervantes et al., 1993b).

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